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Molecular Characterization and Heterologous Expression of Quinate Dehydrogenase Gene from *Gluconobacter oxydans* IFO3244

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Abstract—The quinate dehydrogenase (QDH) from *Gluconobacter oxydans* IFO3244 exhibits high affinity for quinate, suggesting its application in shikimate production. Nucleotide sequence analysis of the *qdh* gene revealed a full-length of 2475-bp encoding an 824-amino acid protein. The *qdh* gene has the unusual TTG translation initiation codon. Conserved regions and a signature sequence for the quinoprotein family were observed. Phylogenetic analysis demonstrated relatedness of QDH from *G. oxydans* to other quinate/shikimate dehydrogenases with the highest similarity (56%) with that of *Acinetobacter calcoaceticus* ADP1 and lower similarity (36%) with a membrane-bound glucose dehydrogenase of *Escherichia coli*. The function of the gene coding for QDH was confirmed by heterologous gene expression in pyrroloquinoline quinone-synthesizing *Pseudomonas putida* HK5.

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Key words: quinate dehydrogenase gene, heterologous gene expression, *Gluconobacter oxydans*, pyrroloquinoline quinone

Shikimic acid and other intermediates in the shikimate pathway can be utilized as building blocks for the production of aromatic amino acids, fine chemicals [1], and pharmaceutical compounds such as the anti-influenza drug GS-4104 [2, 3]. Shikimic acid can be synthesized from glucose using genetically modified strains of *Escherichia coli* [4]. Nonetheless, the biosynthesis pathways in *E. coli* are complicated and require rigorously controlled fermentation conditions to minimize byproduct formation [5]. Alternatively, shikimic acid and the intermediates in the pathway can be produced via the quinate oxidation pathway using acetic acid bacterium *Gluconobacter* strains [6, 7]. Quinate is an abundant natural product that can be oxidized by *Pseudomonas* sp. and *Acinetobacter* sp. and subsequently converted to shikimate via dehydroshikimate. The first enzymatic step in the pathway is the oxidation of quinate, which is catalyzed by quinate dehydrogenase (QDH). QDHs observed in Gram-negative bacteria are generally known to act on either quinate or shikimate; therefore, they have been named quinate/shikimate dehydrogenases. While QDH is an NAD-dependent enzyme in fungi [8], *Aerobacter aerogenes* [9], and *Pseudomonas aeruginosa* [10], it is an NAD(P)-independent enzyme (EC 1.1.99.25) in *Acinetobacter calcoaceticus* [11] and acetic acid bacteria [12]. Purification and characterization of a membrane-bound QDH from *A. calcoaceticus* SA1 [13] and *Gluconobacter oxydans* IFO3244 [14] confirmed the involvement of pyrroloquinoline quinone (PQQ) as a cofactor of the enzyme. It also revealed that PQQ-QDH of *A. calcoaceti-*

Abbreviations: DAS, dense alignment surface method; mGDH, membrane-bound glucose dehydrogenase; PQQ, pyrroloquinoline quinone; QDH, quinate dehydrogenase.

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cus SA1 exhibits similar substrate affinity towards quinate and shikimate, having an apparent K_m of 0.20 and 0.26 mM, respectively. On the other hand, PQQ-QDH from *G. oxydans* IFO3244 showed higher substrate affinity towards quinate than shikimate, having an apparent K_m of 0.19 and 29.7 mM, respectively, suggesting that the enzyme from *G. oxydans* IFO3244 is more appropriate for metabolic engineering of bacteria for shikimate production. Therefore, it is important to perform further genetic investigation of genes involved in the quinate oxidation pathway, i.e. genes coding for PQQ-QDH, dehydroquinate dehydratase, and shikimate dehydrogenase in *G. oxydans* IFO3244.

This report describes the molecular cloning and characterization of the gene encoding PQQ-QDH from *G. oxydans* IFO3244. The primary structure of the deduced amino acids was analyzed and compared with known sequences of PQQ-dependent enzymes. The QDH was heterologously expressed to illustrate the completeness of gene sequence and its function.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation. The bacterial strains and plasmids used in this study are listed in Table 1. The following media were used to cultivate *E. coli* (37°C) [15] and *Pseudomonas putida* HK5 (30°C) cells, i.e. Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl (w/v), pH 7.2) and the basal medium (0.2% $NaNO_3$, 0.2% $(NH_4)_2SO_4$, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.05% yeast extract (w/v), pH 7.0) [16]. The medium and cultivation conditions of *G. oxydans* were as previously described [14]. Importantly, quinate (0.2%, w/v) was provided as an inducer in the medium. In the presence of the plasmid, an appropriate antibiotic was added to the medium to the final concentration of 50 μg/ml, i.e. ampicillin and gentamicin.

DNA isolation, cloning, and sequencing. Extraction of *G. oxydans* IFO3244 genomic DNA and routine molecular techniques were performed as outlined by Sambrook and Russell [17]. Plasmids were prepared using the QIAprep spin miniprep kit (Qiagen, Norway). DNA fragments were purified from agarose gels with the QIAquick gel extraction kit (Qiagen). Restriction enzymes were purchased from NBI Fermentas (Lithuania) and Toyobo (Japan). DNA sequence analyses were performed by Macrogen Inc. (Korea).

PCR amplification, Southern hybridization, inverse PCR, and gene walking. *Gluconobacter oxydans* IFO3244 genomic DNA was used as a template for PCR amplifi-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
Strain		
G. oxydans IFO3244	wild-type strain	IFO*
G. suboxydans IFO12528	wild-type strain	IFO*
P. putida HK5	wild-type strain	[16]
E. coli DH5α	F – *endA1 hsdR*17 *recA*1 *supE*44 *thi-1*ψ80d*lacZ*ΔM15	[15]
Plasmid		
pGEM-T Easy	*E. coli* cloning vector; Amp^r	Promega
pSA19	*Gluconobacter* cloning vector; Amp^r	[36]
pSG8	*Gluconobacter* cloning vector; Amp^r	[37]
pCM62	hybrid of pUC19 and pCM51 (originated from pTJS75, a small IncP plasmid)	[38]
pBBR1-MCS5	broad-host-range cloning vector; Gm^r	[21]
pBBR1-MCS5::*qdh*	pBBR1-MCS5 subcloned with 2536-bp *Sal*I-*Bgl*II restriction fragment of *qdh* gene	this study

Note: Amp, ampicillin; Gm, gentamicin.
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cation of *qdh*. Primers for PCR and inverse PCR are shown in Table 2. For Southern analysis, the genomic DNA was digested for 10 h with restriction enzymes, i.e. *Eco*RI, *Pst*I, *Hin*dIII, *Bam*HI, *Xho*I, *Kpn*I, *Sal*I, *Nar*I, and *Sac*I. The hydrolyzed DNA was separated by agarose gel electrophoresis and transferred onto a Hybond N^+ membrane (Amersham Biosciences, USA) by capillary blotting and was fixed by UV cross-linking [17]. A 1083-bp hybridization probe was PCR-amplified using the primers 231QF and 592QR. The probe was labeled, hybridized, washed, and detected using the ECL (enzyme chemiluminescence) direct nucleotide labeling system (Amersham Biosciences) according to the recommendations of the manufacturer. Subsequently, PCR primers for inverse PCR were designed from the known sequence of *G. oxydans* IFO3244 *qdh* gene fragments. Inverse PCR and gene walking were then performed. DNA sequences were manipulated and assembled using Sequencher 4.8 (Gene Codes Corporation, USA) to obtain a full length *qdh* gene.

Table 2. Oligonucleotides used in this study

Name	Sequence
NF2[a]	5'-AGTCCATCGGCGAAGTCTCC-3'
231QF[b]	5'-GTCTTCCTGTGCACGCCGCATAA-CAA-3'
592QR[b]	5'-GTGCCCGGGGCCGTATACAG-3'
QSacI1_L1[c]	5'-TCGACAACGCTGCCATATCT-3'
QSacI1_R1[c]	5'-GTGGTCGCATTGCTGACAAC-3'
QSacI2_L1[c]	5'-GAGGATGGAAGTGGCGTATT-3'
QSacI2_R1[c]	5'-GTCTACCAGACCGTGCATAA-3'
TF160[d]	5'-CAAGCCATCCTTATtTCCGAAGTG-3'
TR3034[d]	5'-TGAGGCCGCAGGAACGAATGT-TAT-3'
QDH-N1-Sal I[e]	5'-TGTCGACGCCGCAGAACAGGA-GAAACG-3'
QDH-C-Bgl II[e]	5'-TAGATCTCACGGTGAGGCCGCA-GGAAC-3'

[a] Primer designed based on the *N*-terminal sequence of the QDH purified from *G. oxydans* IFO3244 [14].
[b] Primers used to obtain a 1083-bp *qdh* gene fragment that was employed as a probe for Southern hybridization.
[c] Primers used for inverse PCR.
[d] Primers used to obtained a full length *qdh* gene from *G. oxydans* IFO3244.
[e] Primers used to obtained a full length *qdh* gene tagged with a site for restriction digest (underlined) of *Sal*I (*N*-terminus) and *Bgl*II (*C*-terminus).

Analysis of DNA and protein sequence. Sequences were searched for similarity and alignment using BLAST available from the National Center for Biotechnology Information (NCBI) and the ClustalW2 (European Bioinformatics Institute) [18], respectively. The protein domain was analyzed and the phylogenetic tree constructed using the TreeDomViewer [19]. Protein transmembrane segments were analyzed using the dense alignment surface (DAS) method [20].

Construction of pBBR1-MCS5 vector containing the *qdh* gene. The *qdh* gene including the putative ribosome-binding site was amplified from *G. oxydans* IFO3244 chromosomal DNA using PCR primers tagged with *Sal*I restriction site (QDH-N1-*Sal*I) and *Bgl*II restriction site (QDH-C-*Bgl*II) (Table 1). The 2536-bp PCR product was cloned into the pGEM-T Easy cloning vector (Promega, USA). The DNA fragment was then hydrolyzed with *Sal*I and *Bgl*II and cloned into the broad-host range vector pBBR1-MCS5 [21] where the *qdh* gene was arranged co-linear to and downstream of the *lac* promoter. The recombinant plasmid was transformed into *E. coli* DH5α. Transformants were selected on LB-XGal plates supplemented with 50 μg/ml gentamicin. The recombinant plasmid containing the *qdh* gene was named pBBR1-MCS5::*qdh*. The presence of the *qdh* gene was confirmed by restriction mapping and DNA sequencing. The recombinant plasmid, pBBR1-MCS5::*qdh*, was then transformed into *P. putida* HK5 by electroporation.

Heterologous expression of *qdh* gene in *P. putida* HK5. *Pseudomonas putida* HK5 containing pBBR1-MCS5::*qdh* was cultured with 250 rpm-orbital agitation for 18 h, at 30°C in the presence of 50 μg/ml gentamicin in several types of medium, i.e. LB medium or basal medium supplemented either with 0.2% (w/v) quinate, 80 mM glucose, 80 mM fructose, or 80 mM citrate. Cell growth was monitored using a spectrophotometer DU800 (Beckman Coulter, USA) at 600 nm. The cells were harvested by centrifugation at 12,000*g* for 10 min, washed, and resuspended with 50 mM potassium phosphate buffer, pH 7.0. Cells were disrupted using a French pressure cell, the cell debris was removed by low-speed centrifugation, and the cell-free extract was used for enzyme activity assay.

Enzyme assay and protein determination. QDH activity was determined with quinate (5 mM) as a substrate using phenazine methosulfate and 2,6-dichlorophenol-indophenol as the electron acceptors [14]. McIlvaine buffer (pH 6.0 and 8.0) was used. One unit of enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of substrate per minute under the indicated conditions. Protein concentration was measured by a modified Lowry method using bovine serum albumin as the standard protein [22]. The apparent K_m towards quinate and shikimate was determined at pH 8.0 using crude extract of the recombinant *P. putida* HK5.

RESULTS AND DISCUSSION

Molecular cloning and sequence analysis of the quinate dehydrogenase gene of *G. oxydans* IFO3244. Several sets of primers were designed based on the conserved amino acid residues of the gene encoding bacterial quinate dehydrogenase to amplify the *qdh* gene from *G. oxydans* IFO3244. A 1083-bp PCR product was obtained using 231QF and 592QR primers (Table 2) and was then used as a probe for Southern hybridization. To eliminate the possibility of having multiple PQQ-*qdh* paralogs in *G. oxydans* IFO3244, the genomic DNA was digested with several restriction enzymes to create multiple digested patterns. The results of the Southern hybridization suggested that there is no other PQQ-*qdh* gene in *G. oxydans* IFO3244. *Sac*I-digestion of the genomic DNA gave two DNA fragments with amenable size of approximately 4200 and 4800 bp. After inverse PCR of the two DNA fragments followed by gene walking steps and DNA fragment assembly, a full-length *qdh* gene of 2475 bp was obtained. The complete sequence of the *qdh* gene of *G. oxydans* IFO3244 was submitted to GenBank under accession number EU371510. A promoter was not clearly shown upstream of the gene, but the putative sequences, i.e. −35 (TTCATA) and −10 (TCTCATTCT), were proposed at 173 and 153 bp upstream of the translation initiation codon of the *qdh* gene, respectively. The putative Shine–Dalgarno sequence of AGGAGA was observed 12 bp upstream of an unusual TTG translation initiation codon. Although this translation initiation codon is rarely found in *Gluconobacter* sp., it is not unprecedented as it has also been found in *Bacillus* sp. [23, 24].

Analysis of the deduced amino acid sequence of the *qdh* gene (824 amino acid residues (a.a.)) predicted a protein molecular mass of 88.8 kDa [25]. This value agrees with the molecular mass of the purified PQQ-QDH (86 kDa) prior to proteolysis [13, 14, 26]. The deduced amino acid residues at N-terminus (MHSIDQPSSRPAL) depicted partial similarity (underlined) to that of the purified enzyme previously reported (GSSIGEVSSPAGL) [14], although it is not completely identical. This is probably due to an impurity causing a low quality of the data previously obtained from N-terminal amino acid sequencing analysis. The sequence similar to the 21 kDa peptide previously reported was not detectable in the deduced sequence of the gene product. Therefore, it is possible that the 21 kDa peptide previously reported could be a contaminant protein during protein purification. Despite the experimental error on the internal amino acid sequence of the purified QDH, the cloning of the *qdh* gene was achieved and the expression of QDH activity was successfully established confirming its active function as further described.

The deduced amino acid sequence of the *qdh* gene cloned from *G. oxydans* IFO3244 was aligned with other quinate/shikimate dehydrogenase sequences using the ClustalW2 program [18] (Fig. 1). Eight significant sequence motifs, called "W-motifs" (W1-W8), found in a superbarrel structure having eight sets of four anti-parallel β-sheets of PQQ-containing quinoproteins [27] were observed in all six sequences including a membrane-bound glucose dehydrogenase (mGDH) of *E. coli* (Fig. 2). A putative Mg^{2+}-binding site was also noted at amino acid residues 365-367 (ADN) because a magnesium ion is known to be an activator for the enzyme [14]. The QDH sequence of *G. oxydans* IFO3244 exhibited the highest similarity (55-56%) to those of *A. calcoaceticus* ADP1, *X. campestris*, and *P. putida* KT2440. The analysis of protein domain using TreeDomViewer [19], a web-based tool adopting an evolutionary perspective of phylogenetic tree description with PHYLIP format [28] and a protein domain prediction, InterProScan [29], as well as PROSITE, a database of protein families and domains [30] illustrated bacterial quinoprotein dehydrogenase signature 2, W-x(4)-[YF]-D-x(3)-[DN]-[LIVMFYT]-[LIVMFY](3)-x(2)-G-x(2)-[STAG]-[PVT] [31-34], at the amino acid residues 416-437 of the QDH. The phylogenetic analysis (Fig. 2) also confirmed a close relatedness of QDH of *G. oxydans* IFO3244 to that of *A. calcoaceticus* ADP1. The QDH sequence analysis showed a rather fair similarity (36%) to mGDH of *E. coli*. This result is in agreement with a previous report [35] in which an evolutionary relation between PQQ-QDH and mGDH was speculated. Moreover, the analysis of transmembrane segments using the DAS method [20] indicated a similar hydropathy patterns between mGDH [35] and PQQ-QDH (Fig. 3) in which five membrane-spanning regions were predicted near the amino terminus [26].

Expression of the quinate dehydrogenase gene of *G. oxydans* IFO3244 and enzyme activity assay. To illustrate whether the gene cloned from an organism is complete and functional, the gene was cloned into several expression vectors, i.e. an expression vector suitable for acetic acid bacteria, pSA19 [36], an expression vector suitable for *Gluconobacter*, pSG8 [37], and a versatile broad-host range vector for Gram-negative bacteria, pCM62 [38]. The expression vector with the *qdh* gene insertion was transformed back into the original host, *G. oxydans* IFO3244. However, after several intensive transformation procedures, no attempts were successful. Therefore, the *qdh* gene was heterologously expressed. For this purpose, an *E. coli* strain was not chosen as an expression host because *E. coli* strains such as *E. coli* DH5α do not synthesize PQQ, which is the cofactor of QDH. Therefore, gene expression in *E. coli* was avoided. Instead, *G. suboxydans* IFO12528 and *P. putida* HK5 were employed as the hosts for heterologous gene expression of *qdh* from *G. oxydans* IFO3244. The gene encoding QDH was subcloned into pBBR1-MCS5. The pBBR1-MCS5::*qdh* was successfully introduced into

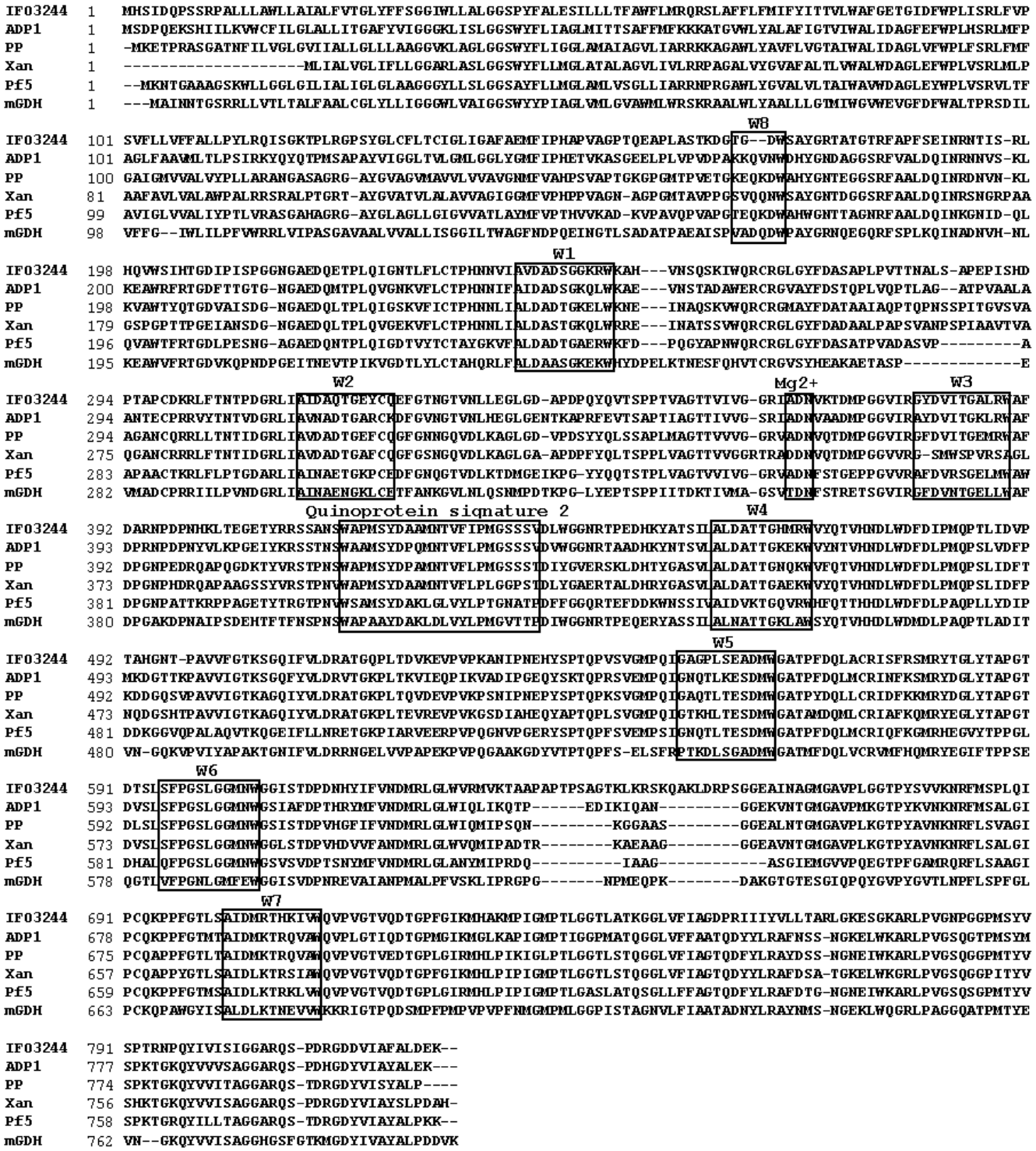

Fig. 1. Alignment of the amino acid sequence of *G. oxydans* IFO3244 QDH and other bacterial quinate/shikimate dehydrogenases. Gene sequences were from *G. oxydans* IFO3244 (IFO3244; GenBank accession No. EU371510), *Acinetobacter* sp. ADP1 (ADP1; GenBank accession No. AAC37161), *P. putida* KT2440 (Pp; GenBank accession No. NP_745706), *Xanthomonas campestris* (Xan; GenBank accession No. AAD38453), *P. fluorescens* Pf-5 (Pf-5; GenBank accession No. YP_262726), and an mGDH of *E. coli* K12 (mGDH; GenBank accession No. NP_414666). W-motifs (W1 to W8), a putative Mg^{2+}-binding site, and a quinoprotein signature sequence 2 are indicated in labeled boxes.

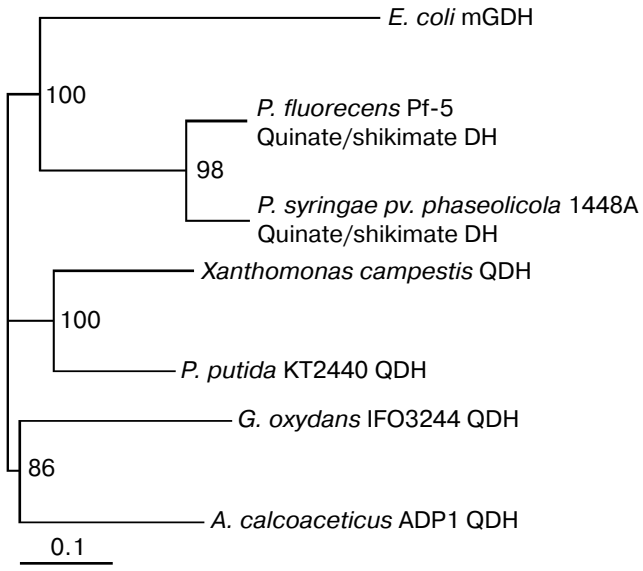

Fig. 2. Phylogenetic relationship among quinate dehydrogenases (for accession numbers see Fig. 1 legend) from various bacteria. The tree topology was inferred by using TreeDomViewer [19]. The scale bar represents 0.1 amino acid substitution per site. The values adjacent to a node indicate the percentage of 1000 bootstrap trees that contain the node.

P. putida HK5, while it failed with *G. suboxydans* IFO12528.

The main concern of *qdh* gene expression in *P. putida* HK5 was whether *P. putida* HK5 might have indigenous quinate/shikimate dehydrogenase activity. Although there are reports on putative gene sequences of quinate/shikimate dehydrogenase in *Pseudomonas* sp., there is no report on the characteristics of the enzyme. In *P. putida* HK5, quinate/shikimate dehydrogenase activity was noticeably induced when cells were grown on the basal-quinate medium (Fig. 4). However, cells grown on LB or the basal-fructose medium exhibited insignificant level of quinate/shikimate dehydrogenase activity, suggesting that the enzyme activity of the host cells was suppressed under these growth conditions. Further study showed that the quinate/shikimate dehydrogenase activity in *P. putida* HK5 wild-type was detected when it was assayed at pH 6.0, but it was completely diminished at pH 8.0 (Fig. 4). On the other hand, the activity of the purified QDH of *G. oxydans* IFO3244 remains at approximately 50% at pH 8.0, while the highest activity was obtained at pH 6.0 [14]. This result demonstrated that the characteristics of the enzymes, i.e. pH dependence of activity, were different. Thus, the QDH activity expressed in *P. putida* HK5 host cells and that of the recombinant plasmid, i.e. pBBR1-MCS5::*qdh*, could be distinguished. Using the assay conditions indicated, QDH activity of *P. putida* HK5 containing pBBR1-MCS5::*qdh* was 0.13 ± 0.01 μmol/min per mg protein, which was approximately 30% of that in *G. oxydans* IFO3244 wild type. Thus, heterologous expression of PQQ-QDH from *G. oxydans* IFO3244 was achieved, although the expression level was rather low.

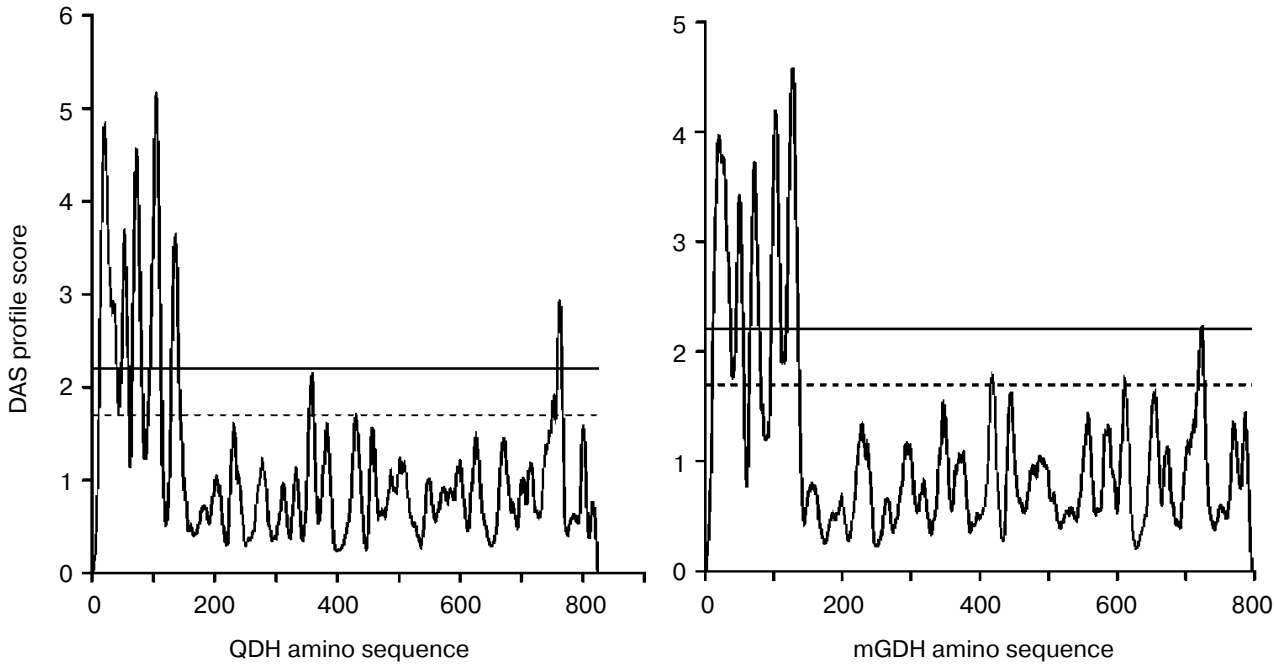

Fig. 3. Putative five transmembrane helices of QDH of *G. oxydans* IFO3244 (824 a.a.) and of mGDH of *E. coli* (796 a.a.). The helices were predicted using the DAS method and are reported as DAS profile score, i.e. loose-cutoff scoring matrix (dashed line) and strict-cutoff scoring matrix (solid line).

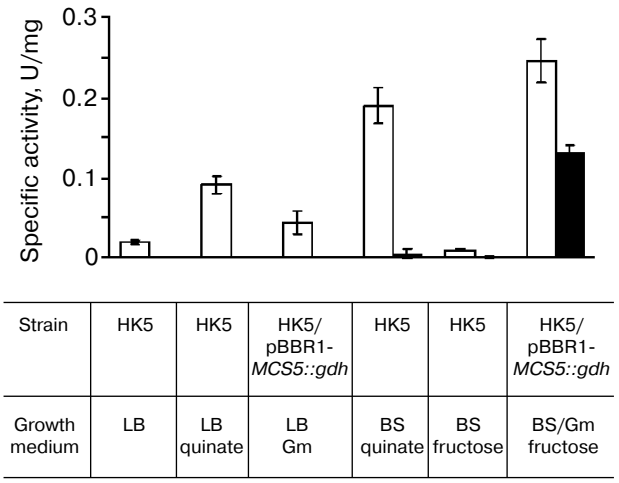

Fig. 4. Heterologous expression of the *qdh* gene in *P. putida* HK5. *Pseudomonas putida* HK5 wild type and the transformant, i.e. *P. putida* HK5 containing pBBR1-MCS5::*qdh*, were grown in various types of growth medium (LB, Luria–Bertani medium; BS, basal medium) in the presence or absence of an inducer, quinate, or other carbon source, e.g. fructose. QDH activity was determined using the phenazine methosulfate-reduction assay at two different buffering pH values: pH 6.0 (opened bar) and pH 8.0 (closed bar). Gentamicin (Gm) was provided only when the transformant was used. Data are means of three individual experiments. Error bars indicate SE.

The apparent K_m values for quinate and shikimate were examined using crude extract of the recombinant *P. putida* HK5. The results indicated that the expressed QDH has low K_m value for quinate (1.0 mM) and much higher K_m value for shikimate (9.5 mM).

In summary, the *qdh* gene was successfully obtained from *G. oxydans* IFO3244. The sequence reported here is a complete, full-length gene. It is a functional gene, which could be expressed in a PQQ-synthesizing host strain such as *P. putida* HK5. The transformant demonstrated a rather fair QDH activity compared to that of the wild-type strain.

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